Chronic hyperplastic anemia as an independent risk factor for atherosclerotic lesions: a lesson from thalassemia intermedia

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Abstract

Introduction. Cardiovascular involvement represents a well-known complication and the primary cause of mortality, both in transfusion-dependent beta thalassemia major (β-TM) and in transfusion-independent beta thalassemia intermedia (β-TI). In β-TM, heart iron overload is considered the main cause of this complication. This is likely due to poor adherence to iron-chelating therapy, resulting in the inability of the body to efficiently remove iron excess derived from transfused red blood cell breakdown. Different clinical pictures may instead be evoked in cardiovascular involvement occurring in β-TI; however, until now, no factor has emerged as the major one responsible for these complications.

Design and Methods. In the present study, iron status, and lipid profiles in serum, as well as lipid content in peripheral blood mononuclear cells (PBMCs) were evaluated in 70 adult β-TM and in 22 adult β-TI patients. Ninety-two age-matched blood donors, free from any form of thalassemia, were utilized as controls. The mRNA levels of genes involved in the regulation of iron metabolism, such as interleukine 1 alfa (IL1α), tumor necrosis factor alfa (TNFα), as well as those involved in cholesterol homeostasis, such as acetyl-coenzymeA: cholesterol acyltransferase (ACAT-1), neutral cholesterol ester hydrolase (nCEH), and ATP binding cassette-A (ABCA1), were also evaluated in PBMCs from the above subjects.

Results. In β-TI patients, serum iron, transferrin saturation and erythropoietin levels were higher, while transferrin and hepcidin were lower, compared to both β-TM and controls. Hepcidin and ILα mRNA levels were found to be reduced in β-TI-PBMCs, while those of TNFα were increased. A reduction in total and high density lipoprotein cholesterol (TC and HDL-C) in serum, and an accumulation of neutral lipids (NL), coupled with increased mRNA levels of ACAT-1 and decreased nCEH in PBMCs were also observed in β-TI.

Conclusions. Since most of the parameters found to be altered in β-TI patients have a key role in the initiation and progression of atherosclerosis, we suggest that cardiovascular complications in these patients may be, at least partially, dependent on the occurrence of premature atherosclerotic lesions.

Key words: thalassemia, iron, cholesterol, atherosclerosis.

Introduction

Beta (β) thalassemia is an inherited hemoglobin disorder characterized by reduced synthesis of β-globin chains. The severity of the clinical course differentiates this heterogeneous disease into three

main subtypes: i) thalassemia major (β-TM) ii) thalassemia intermedia (β-TI) and iii) thalassemia minor (β-TMI).[1] β -TMI is never actively treated, however, although not life threatening on its own, it can affect quality of life due to the effects of a mild to moderate chronic anemia. Patients with β-TM have severe anemia which starts during the first months of life and requires life-long transfusion therapy.[1] Patients with β-TI have a later clinical onset with a milder anemia and, at least for the first decades of their lives, they are usually transfusion-independent.[2] If untreated, both forms of thalassemia are complicated by the multiple effects of chronic hemolytic anemia, such as tissue hypoxia, and by their compensatory reactions, including increased erythropoiesis with bone marrow expansion and increased intestinal iron absorption.[3]

Nowadays, in β-TM patients, due to the early application of regular transfusion-chelation therapy, compensatory reactions induced by hypoxia are strongly reduced. By contrast, in β-TI patients, who did not undergo regular transfusions, tissue-hypoxia persists, and consequently they have bone marrow expansion and increased rates (three to four times the normal ones) of intestinal iron uptake.[4] Cardiovascular complications are a main feature of the clinical spectrum of β-thalassemias. They are the leading cause of death (up to 67%)[5] and have been well documented only in β-TM. The prominent finding in this condition is left ventricle (LV) dysfunction, which is attributed mainly to myocardial overload of iron derived from transfused red blood cell breakdown that gradually leads to cardiac failure and cardiogenic death.[4] Although β-TM and β-TI share common basic pathophysiological mechanisms, cardiovascular involvement may be different in the latter, also considering the fact that no evidence of cardiac iron overload has been found in never-transfused or minimally transfused β-TI patients.[6] Recent studies indicated that endothelial dysfunction, the precipitating factor in the atherosclerotic process, is an important cardiovascular risk determiner in β-TI patients.[7] Therefore, studies aimed at extending the knowledge of the mechanisms connecting atherosclerosis to this type of thalassemia, might help to prevent and/or treat cardiovascular complications in thalassemic subjects.

Among the numerous factors known to confer increased susceptibility to atherosclerosis, iron and cholesterol merit particular consideration in β-TI patients.

It has been demonstrated that the presence of non-transferrin-bound-iron (free iron) in serum generates free radicals which, in turn, cause oxidative vessel injury.[8] Free oxygen radicals act directly on the endothelial cells and have a close interaction with lipid peroxidation, causing a modification of low-density lipoprotein (LDL) and facilitating LDL deposition, with the consequent formation of atherosclerotic plaques.[8]

Accumulating evidence connects hepcidin expression to iron absorption regulation to iron utilization by the erythroid precursors in bone marrow. Hepcidin inhibits duodenal iron absorption as well as iron release from macrophages.[9] Very low mRNA levels of this hormone have been found in hepatic biopsies from β-TI[10]. It has also been reported that interleukin-1 alpha (IL-1α) stimulates hepcidin transcription, indicating this cytokine as a possible iron metabolism regulator. [11]
Hypercholesterolemia is considered one of the most important risk factors in the development of atherosclerosis; however, concentrations of total cholesterol (TC), and LDL-C and/or high-density lipoprotein cholesterol (HDL-C) have been found to be reduced in thalassemic patients[12], those in β-TI being even lower than those in β-TM.[13] These findings make it unlikely that cholesterolemia is responsible for atherogenic risk in these patients.
However, it is important to consider that, besides cholesterolemia, atherosclerosis is associated with profound changes in intracellular lipid metabolism (i.e. monocyte-macrophages cholesterol ester – CE – accumulation).[14]Therefore, before excluding cholesterol as a risk factor for endothelial dysfunction in β-TI patients; cholesterol homeostasis at a cellular level must be investigated.
Starting from these considerations, we measured lipid and iron metabolism in serum and in peripheral blood mononuclear cells (PBMCs) from β-TI and β-TM patients and controls. In addition the mRNA levels of genes controlling iron homeostasis, such as hepcidin, IL-1α and tumor necrosis factor alpha (TNFα), as well as cholesterol homeostasis, such as acetyl-coenzymeA: cholesterol acyltransferase (ACAT-1), the enzyme responsible for cholesterol intracellular esterification, neutral cholesterol ester hydrolase (nCEH), responsible for hydrolysis of CE from cellular storage and ATP binding cassette-A (ABCA1), a mediator of cellular cholesterol efflux, were also determined in PBMCs from enrolled subjects.

Material and Methods

Subjects and blood sampling

Seventy β-TM and twenty-two β-TI adult patients under treatment at the Day Hospital of Ospedale Regionale per le Microcitemie, Centro Talassemici Adulti (Cagliari, Italy) were enrolled. Ninety-two age-matched blood donors, free from any form of thalassemia, enrolled at the Transfusion Center, USL7 (Iglesias, Italy), were utilized as controls. Relevant characteristics of the three groups are summarized in Table 1. All β-TM patients received regular long-term transfusion and iron-chelating therapy. The criteria for the β-TI selection were transfusion independence and lack of a regular iron chelation because of poor adherence or side effects. The study was approved by the local ethics committees and informed consent was given by patients and controls.

Blood samples were collected from each subject and transported on ice at the Department of Biomedical Sciences and Technologies, University of Cagliari (Cagliari, Italy).

Hematologic analysis

Hemoglobin (Hb) was determined by automatic procedures. Serum iron and total iron-binding capacity were measured by using a commercial kit (Sigma-Aldrich). Transferrin saturation was calculated as percentage of serum iron/total iron-binding capacity. Serum ferritin levels were obtained by using an enzyme-linked immunosorbent assay (BioRad); serum erythropoietin and hepcidin were analyzed using commercially available ELISA kits (*R&D Systems Inc*. Minneapolis, United States and DRG Instruments GmbH, Germany, respectively). TC and HDL-C were determined in serum by routine colorimetric enzymatic procedures (Sclavo Diagnostics International S.r.l. Sovicille, Italy).

Isolation of PBMCs

Blood samples were centrifuged at 2200 rpm for 15 min to separate plasma. Plasma was removed and the buffy coat collected. PBMCs were isolated by density gradient centrifugation (Lymphoprep; density, 1.077 g/L; Nycomed Pharma, Oslo, Norway) at 1200 rpm for 10 minutes at 20°C, and washed twice with Hanks balanced salt solution (HBSS) and immediately used for experiments. The trypan blue exclusion test was used to assess cell viability..

Neutral lipid staining

To visualize the degree of cytoplasmic neutral lipid (NL) accumulation, PBMCs were washed three times with PBS, and fixed by soaking in 10% formalin. Cells were then treated with isopropyl alcohol (60%), washed, and stained with oil red O (ORO) (a lipid-soluble dye which stains NL, including CE, but not free cholesterol). They appear as bright red spots in the cytoplasm, and are then counterstained with Mayer's hematoxylin. After staining, cells were imaged using a Leitz inverted-phase microscope fitted with a digital camera. At least two different fields per sample were imaged and analyzed. The red intensity was scored on a semi-quantitative scale (from 0 to 4) by two blinded observers: 0 indicated no staining; 1, rare positive cells or staining barely visible at low power (×200); 2, focal staining or faint diffuse staining clearly visible at low power; 3, multifocal staining or moderate diffuse staining; and 4, intense diffuse staining. There was significantly high correlation between the scores of the two observers (r^2= 0.96; P= 0.000). For convenience, scores from only one observer were used for cell mean-score calculation.

Reverse transcription polymerase chain reaction (RT-PCR) and southern blotting analysis

mRNA levels for genes of interest were evaluated by RT-PCR and Southern blotting. Total RNA was extracted from approximately 10^6 cells using TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA). Equal amounts of total RNA (1 μg) were reverse transcribed into cDNA using the random hexamer method and amplified by PCR in the presence of primers specific for each gene examined, according to the manufacturer's instructions (GeneAmp RNA PCR Kit, Perkin-Elmer Cetus, Norwalk, CT). Preliminary experiments demonstrated that these PCR conditions were within the linear range of amplification of gene fragments. Under our experimental conditions, PCR products separated on agarose and stained with ethidium bromide, showed a major band of the predicted gene size (data not shown). During PCR reaction the non-radioactive label Digoxigenin-11-dUTP (DIG) (Roche Applied Science, Mannheim, Germany) was incorporated and the DNA fragments separated by electrophoresis in agarose were blotted onto a nylon membrane for 16 h in 10×SSC. The blot was exposed to X-ray film (Kodak X-OMAT) for 2–10 min in an X-ray cassette at room temperature. The overall procedure was standardized by expressing the amount of PCR products for the mRNA relative to the amount of product formed for β-actin. The National Institutes of Health Image 1.63 Analysis Software program (Scion Image) was used to assess the intensity of the bands in the autoradiograms.

Statistical analysis

Data were reported as mean ± standard deviation (SD). Statistical analyses were performed with the software Origin (Microcal, Inc, Northampton, MA), and Statistica (StatSoft, Tulsa, OK). Statistical comparisons between the three groups were made by using a one-way ANOVA and where appropriate a post hoc Bonferroni test. A probability of $P < 0.05$ was considered statistically significant.

Results

Iron metabolism parameters of patients and controls

Characteristics of enrolled subjects are shown in Table 1. Sixty-four patients in the β-TM group were anti-HCV positive, while only three in the β-TI one. Erythropoietin levels were higher, and Hb levels lower, in β-TI compared to β-TM and controls.

Table 1. Characteristics of enrolled subjects. Values are expressed as mean ± SD.
***P*=0.016 β-TI *vs* β-TM.**
**** By using the one-way ANOVA test the three groups differ in a statistically significant manner (*P*= 0.000).**
§ Significant differences (*P* < 0.05) by Bonferroni's multiple comparison test.

Subjects	Male	AGE	Anti-HCV positive	Erythropoietin (mU/ml)**	Hb (g/dl)**
Control (n =92)	60	40.7±11.3	0	35.1±9.0	14.8±0.6
β-TM (n=70)	27	38.0±5.0*	64	73.8±57.8§	10.9±0.7§
β-TI (n=22)	14	44.1±8.2*	3	409.8±150.0§	8.5±0.5§

Serum iron status indicators are shown in Table 2**.** Despite the great variability, levels of serum iron and transferrin saturation were significantly higher in β-TI compared to both controls and β-TM. By contrast, transferrin and hepcidin levels were lower in β-TI patients. Serum ferritin levels, which are in equilibrium with the total amount of iron stored in the body, were prominent in β-TM patients. However, they were also significantly higher in β-TI compared to controls.

Table 2. Serum iron status indicators in enrolled subjects. Values are expressed as mean ± SD.
**** By using the one-way ANOVA test the three groups differ in a statistically significant manner (*P* < 0.001).**
**** Significant differences (*P* < 0.05) by Bonferroni's multiple comparison test.**

Subjects	Serum iron* (µg/dl)	Transferrin* test (mg/dl)	Transferrin* saturation (%)	Ferritin* (ng/ml)	Hepcidin* (ng/ml)
Control (n =92)	115. 9±12.0	300.1± 5.0	31.0± 9.0	170.0±20.1	159.7±21.7
β-TM (n=70)	126.3±16.1**	270.2±10.6**	43.7±11.9**	1057.3±800.9**	161.2±33.0**
β-TI (n=22)	170.3±19.2**	163.2±6.8**	70.2±13.0**	504.5±427.1**	117.5±29.1**

Changes in iron status indicators in serum from β-TI patients were associated with altered expressions in PBMCs of genes involved in some way in the regulation of iron homeostasis. In particular, hepcidin and IL-1α mRNA levels were lower and TNFα mRNA was higher in β-TI compared to β-TM and controls.

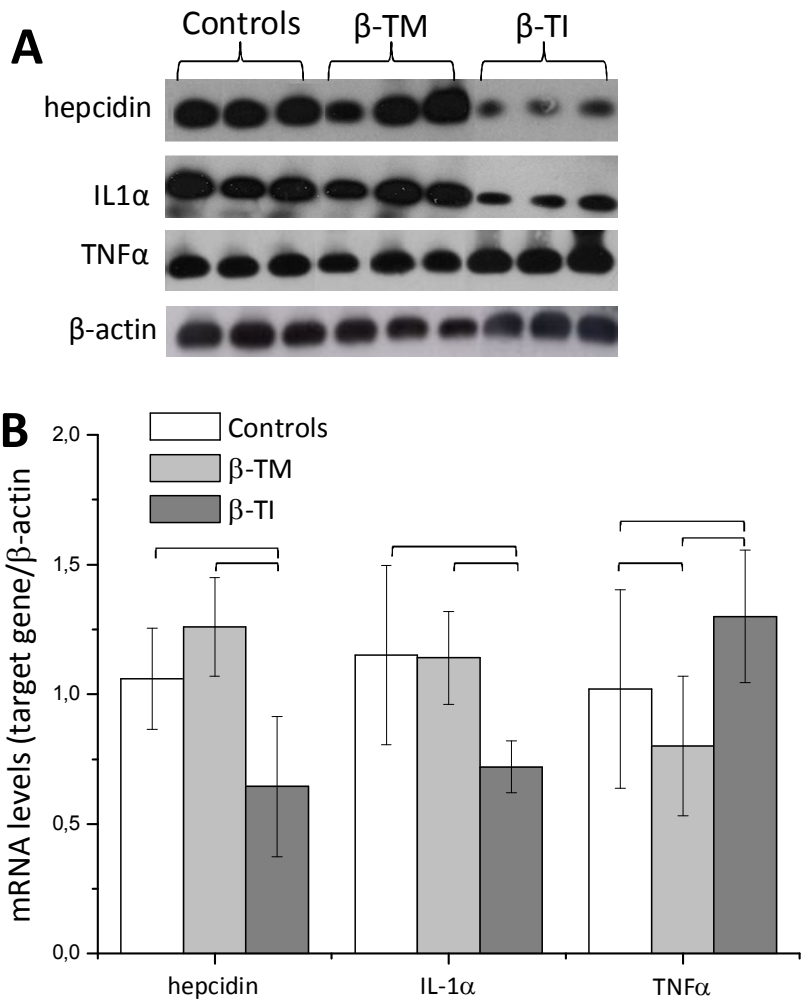

Figure 1. Hepcidin, IL-1α and TNFα mRNA levels in controls, β-TM and β-TI. Total mRNA was extracted from 10^6 unstimulated cells. mRNA levels were determined by RT-PCR using the appropriate primer sets. Specific bands were detected after addition of a chemiluminescent substrate, and analyzed by the NIH Image 1.63 program (Scion Image). (A) Representative blots. (B) Densitometric analysis of mRNA levels, normalized for the endogenous β-actin mRNA Data values are represented as mean ± SD. Statistical analysis performed by using the one-way ANOVA test showed highly significant differences between the groups (P= 0.000). This was followed by Bonferroni's multiple comparison test ($P < 0.05$ between the groups linked by square brackets).

Serum lipid profiles of enrolled subjects

In agreement with studies by other authors [12,13], TC and HDL-C significantly decreased in both β-TM and β-TI. However, by calculating the HDL-C/TC, ratio percentage was significantly lower in β-TI compared to β-TM and controls (Figure 2). These results indicate that, unlike that in β-TM, the TC reduction in β-TI is largely attributable to decreased cholesterol levels in the HDL fraction.

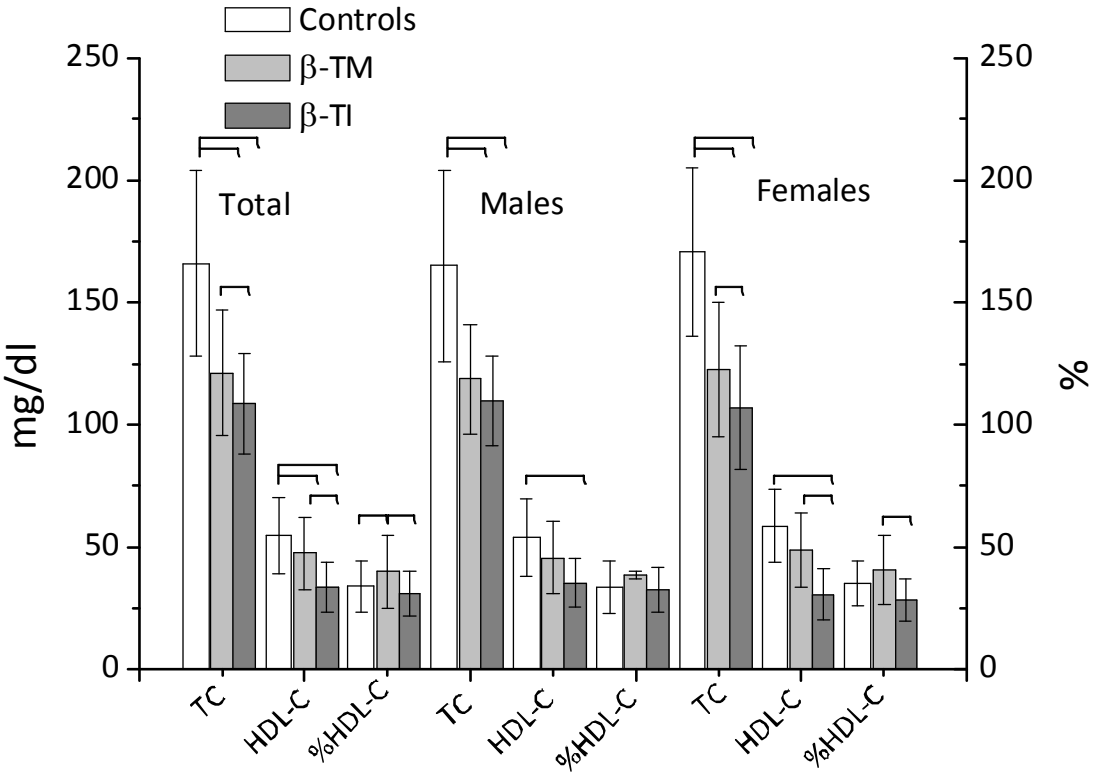

Figure 2. Plasma lipid profile of enrolled subjects. Data are expressed as mean ± SD. Statistical analysis performed by using the one-way ANOVA test showed highly significant differences between the groups (*P*= 0.000). This was followed by Bonferroni's multiple comparison test (*P* < 0.05 between the groups linked by square brackets).

It is well known that depressed HDL-C levels are significantly and independently associated with an increased risk of coronary death.[15,16] This is commonly attributed to the ability of HDL to remove cellular cholesterol excess by interacting with ABCA-1, a transmembrane protein expressed on the surface of cells, including mononuclear blood cells.

In order to verify this possibility we next determined lipid content in PBMCs by staining cells with ORO, a lysochrome fat-soluble dye widely used to demonstrate the presence of NL (mainly CE and triglycerides) which appear as bright red spots in the cytoplasm, as described above in Materials and Methods. As shown in Figures 3A and 3B, NL in control PBMCs were generally absent or very low (mean score 0.6±0.3). PBMCs from β-TM had ORO staining levels scoring 1-2 (mean 1.6±0.8) while those from β-TI had ORO levels scoring 2-4 (mean 2.6±1.3). It is important to note that most unstimulated PBMCs freshly isolated from β-TI have a tendency to form cellular clusters (Figure 3A), hence resembling cultured PBMCs after mitogen activation. This phenomenon was not observed in PBMCs from controls and β-TM (Figure 3A). This finding may have biological significance, to the extent that PBMCs from β-TI patients may be grow-activated *in vivo*.

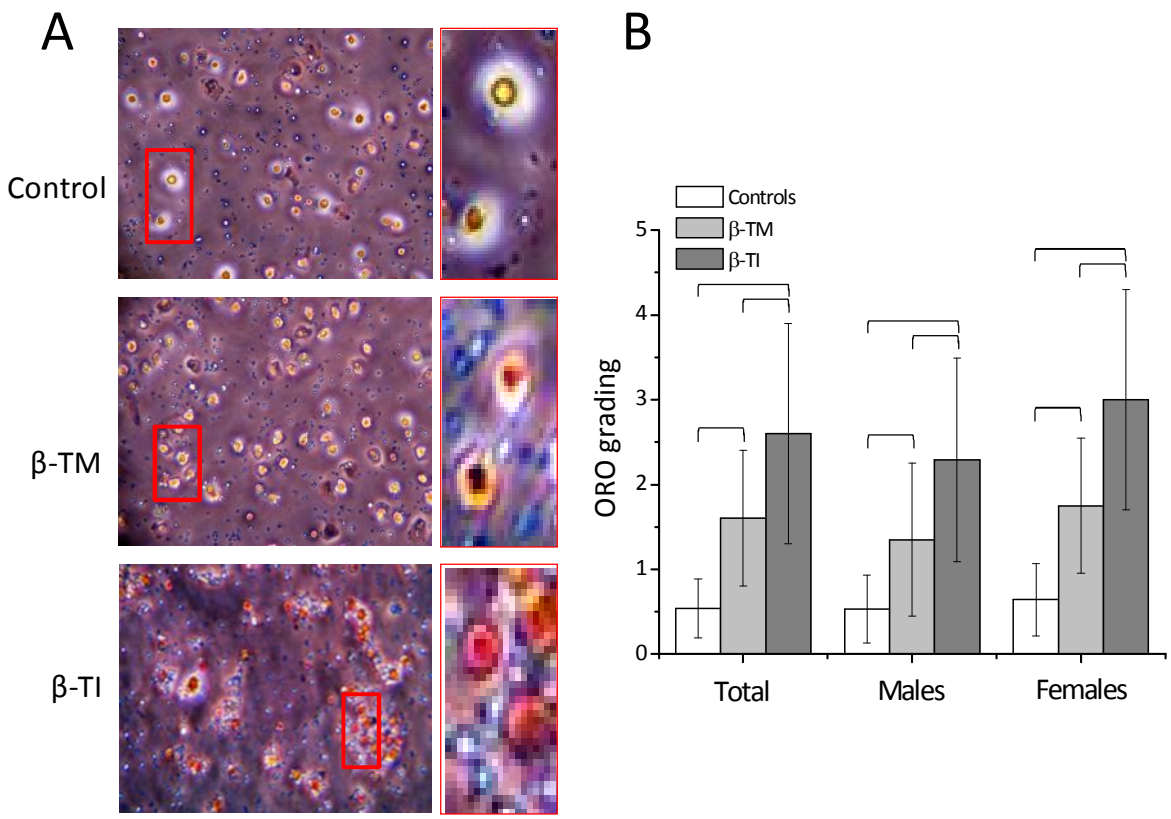

Figure 4. ORO staining in controls, β-TM and β-TI. For these experiments, PBMCs were , immediately after separation, collected, washed and fixed by soaking in 10% formalin, stained with ORO for NL, and counter-stained with Mayer's hematoxylin for nuclei. Cells were then examined by light microscopy and two different fields per sample were imaged. Red ORO intensity was measured in these two fields using NIH Image J software. (A) representative ORO-staining images. (B) ORO grading ± SD. Statistical analysis performed by using the one-way ANOVA test showed highly significant differences between the groups (*P*= 0.000). This was followed by Bonferroni's multiple comparison test (*P* < 0.05 between the groups linked by square brackets).

The above results were further supported by mRNA-level analysis of genes involved in the regulation of cholesterol homeostasis. As shown in Figures 5A and 5B, ACAT-1 mRNA levels significantly increased in both β-TM and β-TI compared to controls. However, ACAT-1 in β-TI was higher than in β-TM. By contrast, nCEH mRNA levels were markedly reduced in β-TI, but not in β-TM. ABCA1 mRNA levels do not vary in β-TM, while slightly decreasing in β-TI.

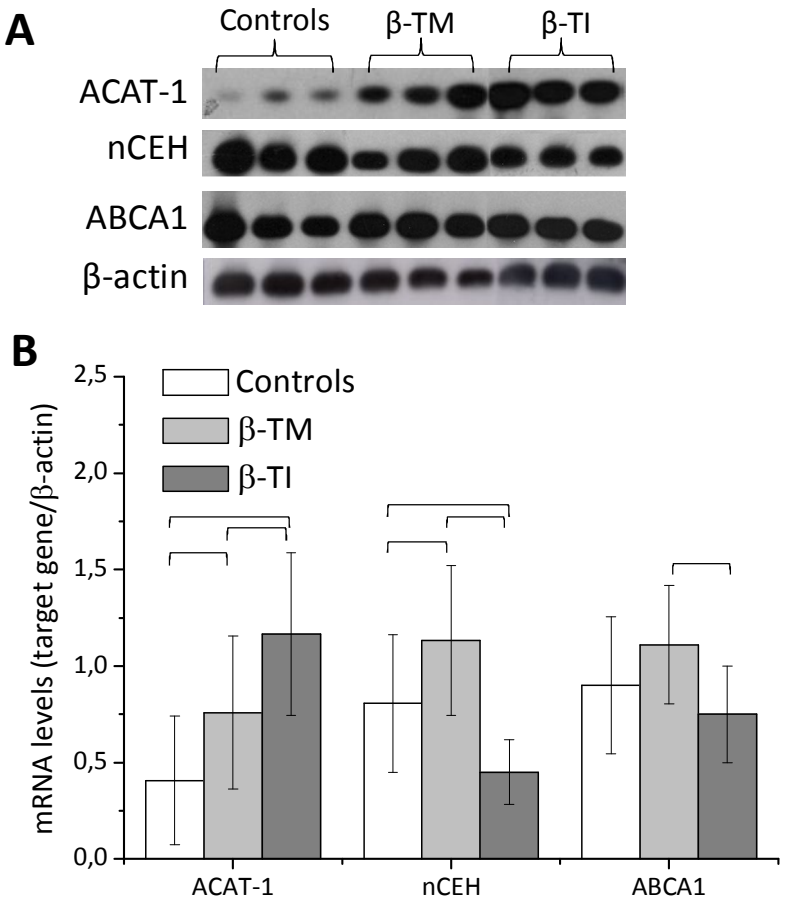

Figure 5 . ACAT-1, nCEH and ABCA1 mRNA levels in controls, β-TM and β-TI. Total mRNA was extracted from 10^6 unstimulated cells. mRNA levels were determined by RT-PCR using the appropriate primer sets. Specific bands were detected after addition of a chemiluminescent substrate, and analyzed by the NIH Image 1.63 program (Scion Image). (A) Representative blots. (B) Densitometric analysis of mRNA levels, normalized for the endogenous β-actin mRNA Data values are represented as mean ± SD. Statistical analysis performed by using the one-way ANOVA test showed highly significant differences between the groups (*P*= 0.000). This was followed by Bonferroni's multiple comparison test (*P* < 0.05 between the groups linked by square brackets).

Discussion

Cardiovascular complications are frequently observed in β-thalassemic patients; nevertheless the pathogenesis of these life-threatening consequences is not yet completely understood.

The data presented here show that long-term transfusions and iron-chelating therapy partially normalizes in β-TM most of the parameters which have been reported to be involved in the pathogenesis of cardiovascular abnormalities in β-thalassemias.[1] In particular, we found that in β-TM, levels of serum iron, transferrin, transferrin saturation, erythropoietin and hepcidin did not greatly differ from control values. Hepcidin and ILα mRNA levels were also comparable to controls in PBMCs from β-TM patients, while TNFα mRNAs were slightly lower. This last finding was not surprising since it has been reported that iron chelators inhibit TNFα expression.[17,18]

However, despite a regular program of transfusions and iron chelating therapy, serum ferritin levels remained high in β-TM patients. Considering that 1 ng/ml of serum ferritin corresponds to about 8 mg of stored iron it can be calculated that β-TM patients have more than eight times organ-stored iron (8.5g) compared to controls (1.3g).[19] These results support the hypothesis that when cardiac dysfunction occurs in β-TM, it is likely due to heart iron overload.

On the other hand, different clinical pictures may be involved in cardiovascular complications occurring in β-TI. In these patients, in fact, serum iron, transferrin saturation and erythropoietin levels were higher, while those of transferrin and hepcidin were lower compared to both β-TM and controls. Hepcidin and ILα mRNA levels were found to be lower in β-TI-PBMCs, while those of TNFα mRNA were higher. These results indicate that in β-TI, low ILα levels, coupled with persistent bone marrow hyperplasia, secondary to ineffective erythropoiesis-dependent anemia, bring about inappropriate chronic hepcidin suppression. Loss of functioning hepcidin raises serum iron levels, decreases reticulo-endothelial iron stores, and increases intestinal iron absorption.[9,10] Therefore the consequent free serum iron increase may induce lipid peroxidation, endothelial cell activation, and reactive oxygen species generation, all of which are considered important early events in the pathogenesis of atherosclerosis.[20]

Hypocholesterolemia has been frequently described in thalassemic subjects, and for this reason a lower incidence of atherosclerotic events in these subjects has even been suggested.[21,22] In spite of this, the exact mechanism/s and the possible clinical consequences of hypocholesterolemia in thalassemic patients have not yet been elucidated. It has been recently demonstrated that patients with chronic anemia and increased erythropoietic activity have hypocholesterolemia, whereas those with low erythropoietic activity are normo-cholesterolemic,[23] suggesting that in the former hypocholesterolemia is related to a higher cholesterol requirement by erythroid cells in order to sustain cellular growth. In the present study we confirmed that patients with β-TI had significantly lower total and HDL cholesterol, not only compared to controls but also compared with β-TM patients. Additionally, we also showed that PBMCs isolated from β-TI patients accumulated more neutral lipids, mainly CE, in their cytoplasm, compared to PBMCs from β-TM and controls. RT-PCR analysis also revealed that ACAT-1 mRNA levels were significantly higher in β-TI, compared to β-TM, while nCEH mRNA levels were slightly reduced. These data clearly indicate that, in β-TI, bone marrow progenitor cells entrapped more neutral lipids to sustain active growth. As a consequence, total and HDL cholesterol may decrease.

Although further studies are needed in order to fully understand the pathogenesis of cardiovascular disorders in thalassemic patients, the results of the present study offer us a way to speculate on the

possible mechanism/s by which thalassemic patients not only are not protected against atherosclerosis, but may even be considered at a higher risk of developing this severe condition.

It is well known that transendothelial migration of monocytes and lymphocytes is a fundamental mechanism in atherogenesis,[20] this process being partly mediated by the interaction of carbohydrate ligands present on leukocyte membranes with adhesion molecules[24] present on the surface of the activated endothelium cells.

NMR spectroscopy studies have shown that the majority of non-transferrin-bound iron (NTBI) in the plasma of iron-overloaded patients is present as Fe^{3+} complexes with citrate.[25] Using this iron complex as the source of free iron in the incubation medium, Kartikasari et al.,[26] showed that 10μmol/L of iron directly activates and stimulates the expression of adhesion molecules on both umbilical-vein endothelial cells and monocytes, as well as promoting monocyte adhesion to endothelial cells. It has also been demonstrated that up-regulation of TNFα enhances adhesion molecule expression in epithelial cells [27]

β-TI patients enrolled for this study exhibited high plasma transferrin saturation as well as high TNFα mRNA levels in their PBMCs, indicative of chronic endothelial activation. In addition, accumulation of CE, the main components of atherosclerotic plaque fatty lesions, was also found in mononuclear cells from these patients. Hence it may be supposed that, in β-TI patients, high free serum iron levels, besides causing a direct toxic effect through oxygen radical production, may also play an independent role in promoting leukocyte recruitment to the endothelium. As a result, CE-rich leukocytes migrate into vessel walls, thus accelerating the atherosclerotic process. These conclusions fit well with the finding that β-TI-PBMCs tend to form clusters in vitro, which is indicative of growth activation in vivo. If this hypothesis is correct, it may be argued that any chronic anemia associated with high-erythropoietic activity including silent carrier state (β-TMI), which in Sardinia affects about 12% of the population, may increase the risk of developing atherosclerosis. Accordingly, Sarnak et al.[28] found in a study cohort (14,410 subjects total) significant increase of cardiovascular disease in chronic anemic subjects.

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References

1. Rund D, Rachmilewitz E. Beta-thalassemia. N Engl J Med 2005;353: 1135-46.
2. Camaschella C, Cappellini MD. Thalassemia intermedia. Haematologica 1995;80: 58-68.
3. Aessopos A, Farmakis D, Karagiorga M, Voskaridou E, Loutradi A, Hatziliami A, et al. Cardiac Involvement in Thalassemia Intermedia: a Multi-center Study. Blood 2001;97: 3411-6.

4. Aessopos A, Kati M, Farmakis D. Heart disease in thalassemia intermedia: a review of the underlying pathophysiology. Haematologica 2007;92: 658-65.
5. Borgna-Pignatti C, Rugolotto S, De Stefano P, Zhao H, Cappellini MD, Del Vecchio GC, et al. Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. Haematologica 2004;89: 1187-93.
6. Origa R, Barella S, Argiolas GM, Bina P, Agus A, Galanello R. No evidence of cardiac iron in 20 never- or minimally-transfused patients with thalassemia intermedia. Haematologica 2008;93: 1095-6.
7. Aessopos A, Tsironi M, Andreopoulos A, Farmakis D. Heart Disease in Thalassemia Intermedia. Hemoglobin 2009;33: 170-6.
8. Auer JW, Berent R, Weber T, Eber B. Iron Metabolism and Development of Atherosclerosis. Circulation 2002;106: 7.
9. Ganz T. Hepcidin—a regulator of intestinal iron absorption and iron recycling by macrophages. Best Prac Res Clin Haematol 2005;18: 171-82.
10. Camberlein E, Zanninelli G, Détivaud L, Lizzi AR, Sorrentino F, Vacquer S, at al. Anemia in β-thalassemia patients targets hepatic hepcidin transcript levels independently of iron metabolism genes controlling hepcidin expression Haematologica 2008;93: 111-15.
11. Lee P, Peng H, Gelbart T, Wang L, Beutler E. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. PNAS 2005;102: 1906-10.
12. Nasr M, Abdelmaksoud A, Abd El-Aal K, Mabrouk N, Ismael W. Plasma lipid profile and lipid peroxidation in beta-thalassemic children. J Clin Lipidol 2008; 6: 405-9.
13. Amendola G, Danise P, Todisco N, D'Urzo G, Di Palma A, Di Concilio R. Lipid profile in beta-thalassemia intermedia patients: correlation with erythroid bone marrow activity. Int J Lab Hematol 2007;29:172-6.
14. Tiwari RL, Singh V, and Barthwal MK. Macrophages: An elusive yet emerging therapeutic target of atherosclerosis. Med Res Rev 2008;28: 483-544.
15. Wilson PW, Abbott RD, Castelli WP. High density lipoprotein cholesterol and mortality. The Framingham Heart Study. *Arteriosclerosis* 1988;8:737-41.
16. Goldbourt U, Yaari S, Medalie JH. Isolated low HDL cholesterol as a risk factor for coronary heart disease mortality. A 21-year follow-up of 8000 men. Arterioscler Thromb Vasc Biol 1997;17:107-13.
17. Zhang WJ, Frei B. Intracellular metal ion chelators inhibit TNFα induced SP-1 activation and adhesion molecule expression in human aortic endothelial cells. Free Radic Biol Med 2003;34: 674-82.

18. Koo SW, Casper KA, Otto KB, Gira AK, Swerlick RA. Iron chelators inhibit VCAM-1 expression in human dermal microvascular endothelial cells. J Invest Dermatol 2003;120: 871-879.
19. Walters GO, Miller FM, Worwood M. Serum ferritin concentration and iron stores in normal subjects. J Clin Pathol 1973;26: 770-2.
20. Steinberg D. Thematic review series: The Pathogenesis of Atherosclerosis. An interpretive history of the cholesterol controversy, part III: mechanistically defining the role of hyperlipidemia. J Lipid Res 2005;46: 2037-51.
21. Calandra S, Bertolini S, Pes GM, Deiana L, Tarugi P, Pisciotta L, et al. Beta-thalassemia is a modifying factor of the clinical expression of familial hypercholesterolemia. Semin Vasc Med 2004;4: 271-8.
22. Namazi M. Minor thalassemia as a protective factor against cerebrovascular accidents Medical Hypotheses 2002; 59: 361-2.
23. Shalev H, Kapelushnik J, Moser A, Knobler H, Tamary H. Hypocholesterolemia in chronic anemias with increased erythropoietic activity. Am J Hematol 2006**;** 82: 199-202.
24. Bevilacqua MP, Nelson RM. Selectins. J Clin Invest 1993; 91: 379-87.
25. Grootveld M, Bell JD, Halliwell B, Aruoma OI, Bomford A, Sadler PJ. Non–transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. J Biol Chem 1989; 264: 4417-22.
26. Kartikasari AER, Georgiou NA, Visseren FLJ, van Kats-Renaud H, Sweder van Asbeck B, Marx JJM**.** Intracellular Labile Iron Modulates Adhesion of Human Monocytes to Human Endothelial Cells. Arter Thromb Vasc Biol 2004;24: 2257-62.
27. Osborn L, Hession C, Tizard R, Vassallo C, Luhowskyj S, Chi-Rosso G, Lobb R. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 1989**;**59: 1203–11.
28. Sarnak MJ, Tighiouart H, Manjunath G, MacLeod B, Griffith J, Salem D, et al. Anemia as a Risk Factor for Cardiovascular Disease in the Atherosclerosis Risk in Communities (ARIC) Study. J Am Coll Cardiol 2002;40: 27-33.